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Functional Consequences of Subunit Diversity in RNA Polymerases II and V

Ek Han Tan,[1,3] Todd Blevins,[1] Thomas S. Ream,[3,4] and Craig S. Pikaard[1,2,*]
[1]Department of Biology and Department of Molecular and Cellular Biochemistry
[2]Howard Hughes Medical Institute
Indiana University, Bloomington, IN 47405, USA
[3]Division of Biology and Biomedical Sciences, Washington University, St. Louis, MO 63110, USA
[4]Department of Biochemistry, University of Wisconsin, Madison, WI 53706, USA
*Correspondence: cpikaard@indiana.edu
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SUMMARY

Multisubunit RNA polymerases IV and V (Pol IV and Pol V) evolved as specialized forms of Pol II that mediate RNA-directed DNA methylation (RdDM) and transcriptional silencing of transposons, viruses, and endogenous repeats in plants. Among the subunits common to *Arabidopsis thaliana* Pols II, IV, and V are 93% identical alternative ninth subunits, NRP(B/D/E)9a and NRP(B/D/E)9b. The 9a and 9b subunit variants are incompletely redundant with respect to Pol II; whereas double mutants are embryo lethal, single mutants are viable, yet phenotypically distinct. Likewise, 9a or 9b can associate with Pols IV or V but RNA-directed DNA methylation is impaired only in *9b* mutants. Based on genetic and molecular tests, we attribute the defect in RdDM to impaired Pol V function. Collectively, our results reveal a role for the ninth subunit in RNA silencing and demonstrate that subunit diversity generates functionally distinct subtypes of RNA polymerases II and V.

INTRODUCTION

Eukaryotes decode their genomes using three essential nuclear DNA-dependent RNA polymerases, RNA Polymerases I, II, and III (abbreviated as Pol I, Pol II, and Pol III) (Cramer et al., 2008; Werner and Grohmann, 2011). In plants, two additional multisubunit RNA polymerases, Pol IV and Pol V, are not strictly required for viability but are important for development, transposon taming, antiviral and antibacterial defense, and interallelic communications mediating paramutation (Haag and Pikaard, 2011).

Pol IV and Pol V functions are best understood with respect to RNA-directed DNA methylation (Haag and Pikaard, 2011; Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005), a process in which 24 nt short interfering RNAs (siRNAs) direct the cytosine methylation, and silencing, of complementary DNA sequences. Pol IV acts early in the pathway, working in partnership with RNA-DEPENDENT RNA POLYMERASE 2 to produce double-stranded RNAs that are diced into siRNAs and loaded (primarily) into ARGONAUTE 4 (AGO4) (Law and Jacobsen, 2010). Independent of siRNA biogenesis, Pol V generates RNA transcripts at loci that undergo RdDM (Wierzbicki et al., 2008) and AGO4 binds these Pol V transcripts (Wierzbicki et al., 2009) as well as Pol V itself (El-Shami et al., 2007). Chromatin modifying activities are subsequently recruited, resulting in de novo cytosine methylation and establishment of repressive histone modifications (Haag and Pikaard, 2011; Law and Jacobsen, 2010).

Arabidopsis thaliana Pols II, IV, and V each have 12 core subunits (Ream et al., 2009). Pol II, IV, and V largest subunits are encoded by unique genes: *NRPB1*, *NRPD1*, and *NRPE1*, respectively ("NRP" denotes "Nuclear RNA Polymerase"; "B, D, and E," as the second, fourth, and fifth letters of the alphabet, denote Pols II, IV, or V; the numeral 1 indicates the largest subunit). Pol IV and V second-largest subunits are encoded by the same gene, *NRP(D/E)2*, which is distinct from the corresponding Pol II subunit gene, *NRPB2* (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005). The two largest subunits interact to form the catalytic center for RNA synthesis, with noncatalytic subunits playing structural and regulatory roles for initiation, elongation, termination, or RNA processing (Cramer et al., 2008; Werner and Grohmann, 2011). Most of the noncatalytic subunits of Pols II, IV, and V are encoded by the same genes (Ream et al., 2009).

In yeast, the 12 Pol II subunits are each encoded by unique genes, ten of which are essential. One of the nonessential genes encodes the ninth-largest subunit, Rpb9. *rpb9* deletion strains are viable, but temperature-sensitive (Woychik et al., 1991). Rpb9 is implicated in multiple aspects of polymerase II function, including initiation (Hull et al., 1995; Sun et al., 1996), processivity (Awrey et al., 1997; Hemming et al., 2000), transcriptional fidelity, proofreading (Koyama et al., 2007; Nesser et al., 2006; Walmacq et al., 2009), and transcription-coupled DNA repair (Li et al., 2006).

Unlike yeast and metazoans, *Arabidopsis thaliana* and *Populus trichocarpa* (poplar) each have two genes orthologous to *RBP9*, and maize and rice have three (Figure 1 and Figure S1). Orthologs of yeast Rpa12 and Rpc11, the Rpb9-like subunits of Pols I and III, respectively, form separate clades (Figure 1). Both *Arabidopsis* Rpb9 orthologs copurify with affinity-purified RNA polymerases II, IV, or V (Law et al., 2011; Ream et al., 2009),

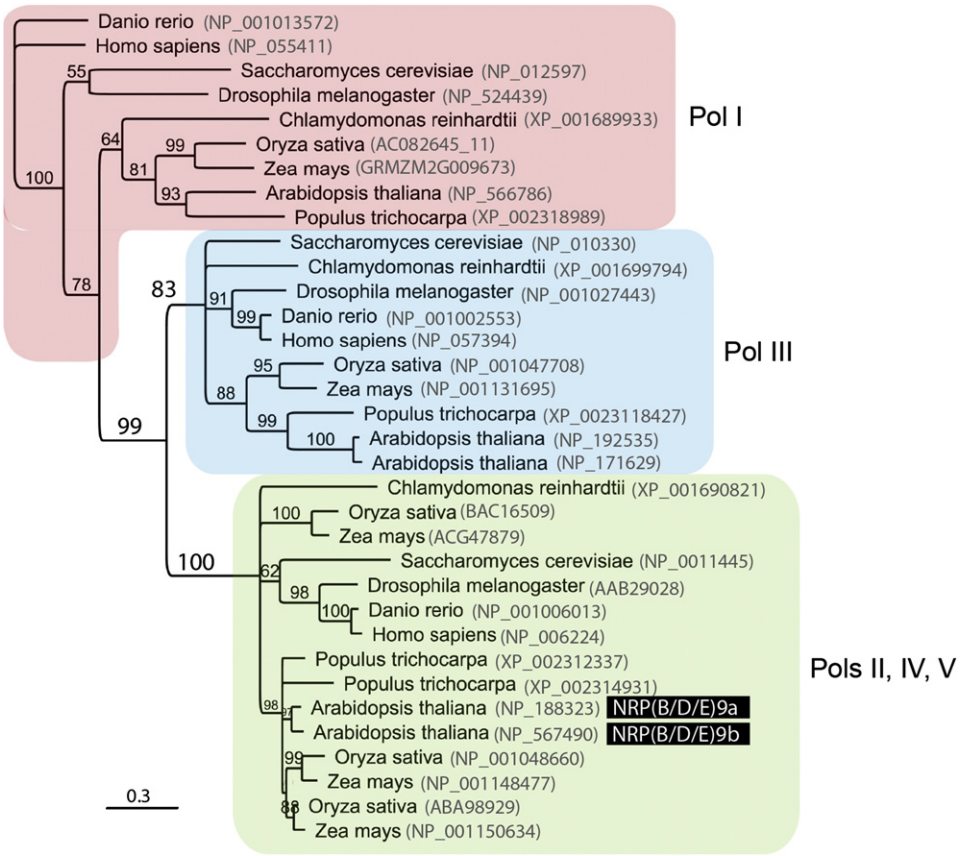

Figure 1. Phylogenetic Relationships among Eukaryotic RNA Polymerase Subunits Homologous to Yeast Rpb9

Human, *Drosophila*, zebrafish, *Chlamydomonas*, *Arabidopsis*, poplar, maize, or rice proteins homologous to yeast (*S. cerevisiae*) Rpb9 (Pol II), or its Pol I or Pol III paralogs, were identified by searching the NCBI Reference Sequence (RefSeq) or maizeGBD (in the case of *Zea mays* GRMZM2G009673) databases using BLASTp. Multiple alignment of the sequences was conducted using MUSCLE (see Figure S1) in order to generate the phylogenetic tree. Bootstrap values are indicated for each branchpoint.

such that their comprehensive names are NRP(B/D/E)9a and NRP(B/D/E)9b. Despite the fact that the two proteins differ at only 8 of their 114 amino acids, we show here that these ninth subunit variants are incompletely redundant for Pol II and nonredundant for Pol V functions.

RESULTS

Subunits 9a and 9b Are Redundant for Viability

Arabidopsis NRP(B/D/E)9a (*At3g16980*) and *NRP(B/D/E)9b* (*At4g16265*) genes have similar intron/exon structures (Figure 2A). T-DNA insertion alleles, designated *nrp(b/d/e)9a-1* (Salk_032670) and *nrp(b/d/e)9b-1* (Salk_031043), are disrupted within introns 2 or 1, respectively (Figure 2A). Transcripts of the *9a* and *9b* (abbreviated for brevity) genes are readily detected in wild-type plants (Figure 2B) but not in *9a-1* or *9b-1* mutants (Figure 2C).

Wild-type (ecotype Col-0) and *9a-1* mutant plants are indistinguishable, but leaves of *9b-1* mutants are more ovate, have shorter petioles and display less downward edge curling (Figure 2C). Other *9b-1* phenotypes include smaller trichomes on the first true leaves, more prominent leaf midveins, changes in the cuticular wax coating on the leaves, and shorter siliques. These morphological differences presumably result from altered Pol II-dependent gene expression given that null mutations eliminating Pol IV or Pol V largest subunits (*nrpd1-3* or *nrpe1-11*, respectively) do not induce similar phenotypes. Moreover, *9b-1* phenotypes are neither suppressed nor enhanced in *9b-1 nrpd1-3* or *9b-1 nrpe1-11* double mutants.

To test *9a* and *9b* redundancy, homozygous *9a-1* and *9b-1* mutants were crossed, resulting F1 plants were selfed, and their progeny genotyped. In siliques of F2 plants homozygous for *9a-1* and heterozygous for *9b-1*, in which 25% of the F3 seeds are expected to be homozygous *9a-1 9b-1* double mutants, 30% (55/181 analyzed) of the seeds arrested in development and 70% developed normally (Figure 2D). Similar results were observed for the progeny of plants homozygous for *9b-1* but heterozygous for *9a-1*. In arrested seeds, which are translucent, embryos failed to develop past the globular stage (Figure 2D). Among plants germinated from seeds collected from siliques of plants that were homozygous for either *9a-1* or *9b-1* and heterozygous for the other mutation, no *9a-1 9b-1* double mutants were

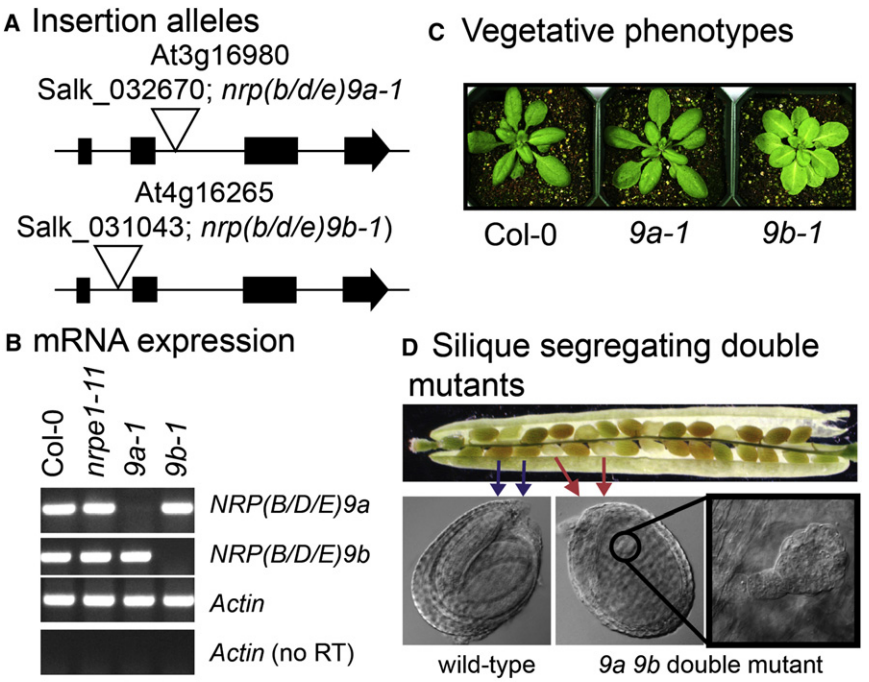

Figure 2. NRP(B/D/E)9a and 9b Are Redundant for Viability

(A) Positions of T-DNA insertions within the *nrp(b/d/e)9a-1* and *nrp(b/d/e)9b-1* alleles are indicated by triangles. Filled boxes represent exons; lines within boxes represent introns. Genes At3g16980 and At4g16265 encode the 9a and 9b protein sequences whose accession numbers (shown in Figure 1 and Figure S1) are NP_188323 and NP_567490, respectively.

(B) RT-PCR amplification of *NRP(B/D/E)9a*, *NRP(B/D/E)9b* or actin mRNAs in wild-type (Col-0), Pol V largest subunit null mutant (*nrpe1-11*), or *9a-1* or *9b-1* mutant plants. Actin RT-PCR reactions in which reverse transcriptase was omitted were also conducted (bottom row) to assess potential DNA contamination of the RNA samples.

(C) Vegetative phenotypes of 3-week-old wild-type (Col-0), *9a-1* mutant or *9b-1* mutant plants.

(D) Ovules within a silique of a plant homozygous for *9a-1* and heterozygous for *9b-1* (upper image). Opaque ovules (blue arrows) contain fully developed embryos whose primary roots and (folded-over) cotyledons can be observed by differential interference microscopy (image at lower left). By contrast, translucent ovules (red arrows), occurring at the expected frequency of *9a-1 9b-1* double mutants, lack mature embryos (bottom center and bottom right images). The image at bottom right shows a blow-up of the region circled in the bottom center image, revealing an embryo arrested at the globular stage of development.

(E) Homozygous *9a-1 9b-1* double mutants are not recovered among seedling progeny of plants homozygous for *9a-1* or *9b-1* and heterozygous for the other allele. Heterozygote under-representation results from reduced transmission of mutant alleles via both the male and female gametophytes (see Figure S2).

E Mutant allele transmission

Parent genotype	Progeny genotypes and their frequency		
9a -/- , 9b +/-	*9a -/- , 9b -/-*	*9a -/- , 9b +/-*	*9a -/- , 9b +/+*
	0	69	60

Parent genotype	Progeny genotypes and their frequency		
9a +/- , 9b -/-	*9a -/- , 9b -/-*	*9a +/- , 9b -/-*	*9a +/+ , 9b -/-*
	0	61	68

identified (Figure 2E). Because lethality is a consequence of lost Pol II function (Onodera et al., 2008), but not disrupted Pol IV or Pol V function (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005; Ream et al., 2009), we conclude that the 9a and 9b subunits are mostly redundant with respect to Pol II functions required for viability and development, such that only the double mutant is embryo lethal.

Among the progeny of heterozygotes carrying a recessive allele of an essential gene, heterozygotes should outnumber homozygotes 2:1. However, we observed a nearly 1:1 ratio of heterozygotes to homozygous wild-type plants (Figure 2E), suggesting a defect in male or female (or both) transmission of the mutant alleles. Reciprocal crosses showed reduced transmission of *9a-1* or *9b-1* alleles through both the male and female gametophytes (Figure S2). This allele transmission behavior differs from null mutations eliminating catalytic subunits of Pols I, II, or III, which show zero transmission via the egg donor (Onodera et al., 2008).

Subunit 9b Is Required for RNA Silencing

Loci silenced by Pol IV and Pol V include soloLTR and AtSN1 retroelements, whose expression is undetectable in wild-type plants (Col-0) but prevalent in *nrpd1-3* (Pol IV largest subunit) or *nrpe1-11* (Pol V largest subunit) mutants (Figure 3A). In *9a-1* mutants, soloLTR and AtSN1 silencing is unaffected. However, these retroelements are derepressed in *9b-1* mutants (Figure 3A).

Retrotransposon silencing correlates with Pol IV and Pol V-dependent RNA-directed DNA methylation (RdDM), rendering methylation-sensitive *Alu*I sites within *soloLTR* elements and HaeIII sites within *AtSN1* elements resistant to *Alu*I or HaeIII digestion. Consequently, PCR using primers that flank the restriction sites amplifies the interval in wild-type (Col-0) plants (Figure 3B). However, in *nrpd1-3* or *nrpe1-11* mutants, loss of RdDM allows *Alu*I and HaeIII to cleave the DNA, and PCR fails (Figure 3B). Methylation is similarly lost in *9b-1* mutants, but not in *9a-1* mutants (Figure 3B).

Tandemly repeated 5S ribosomal RNA genes are also subject to RdDM. Southern blots of genomic DNA digested with HaeIII and hybridized to a 5S rRNA gene probe show a ladder of bands in wild-type Col-0 plants (Figure 3C), with larger bands reflecting increased methylation among adjacent genes. HaeIII methylation is reduced in *nrpd1-3*, *nrpe1-1*, and *9b-1* mutants, but not in *9a-1* mutants.

A complete *NRP(B/D/E)9b* transgene, transcribed from its native promoter and containing all its introns, rescues all *9b-1*

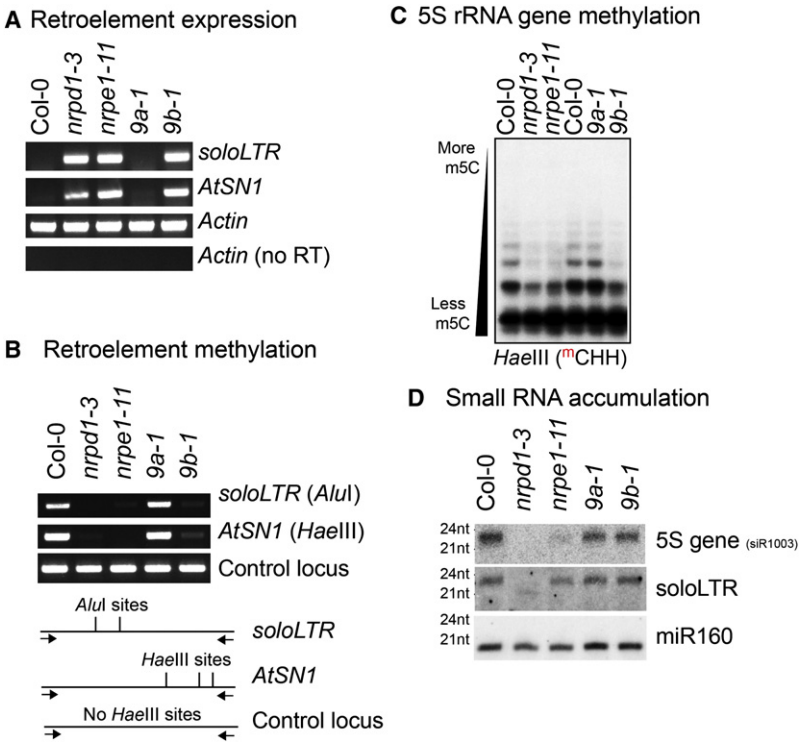

Figure 3. *9b-1* Mutants Are Defective for RNA-Directed DNA Methylation and Silencing

(A) RT-PCR analysis of soloLTR and AtSN1 retroelement expression, comparing Pol IV and Pol V largest subunit null mutants, *nrpd1-3* and *nrpe1-11*, respectively, with *9a-1*, *9b-1*, and wild-type (Col-0). The loss of silencing in the *9b-1* mutant is restored by a full-length *9b* transgene (see Figure S3).

(B) Cytosine methylation assay involving PCR amplification of soloLTR and AtSN1 retrotransposons following incubation of genomic DNA with the methylation sensitive restriction endonucleases, HaeIII or *Alu*I. The control locus, *At2g19920,* lacks HaeIII sites. Diagrams show positions of restriction endonuclease recognition sites within the amplicon.

(C) Southern blot analysis of tandemly repeated 5S rRNA genes following digestion of genomic DNA with HaeIII.

(D) RNA blot hybridization analysis of inflorescence small RNAs using 5S rRNA gene (siR1003), soloLTR, and microRNA (miR160) probes. The same blot was stripped and rehybridized sequentially. miR160 serves as an RNA loading control.

mutant phenotypes, restoring wild-type leaf morphologies (Figure S3A), *NRP(B/D/E)9b* mRNA production (Figure S3B), *AtSN1* and *soloLTR* silencing (Figure S3B), and cytosine methylation (Figure S3C). We conclude that *9b-1* mutant phenotypes are due solely to mutation of the *NRP(B/D/E)9b* gene.

Pol IV Function Is Not Impaired in 9b-1 Mutants

Pol IV is required for the biogenesis of ~95% of all 24 nt siRNAs (Herr et al., 2005; Kanno et al., 2005; Mosher et al., 2008; Onodera et al., 2005; Pontier et al., 2005; Zhang et al., 2007). For instance, 24 nt siRNAs corresponding to 5S rRNA genes and soloLTR retrotransposons are severely depleted in *nrpd1-3* mutants (Figure 3D) (Onodera et al., 2005). In *9a-1* or *9b-1* mutants, siRNAs are detected at wild-type levels, suggesting that Pol IV activity is not impaired. Interestingly, reductions in siRNA levels observed in *nrpe1* mutants are also not apparent in *9a-1* or *9b-1* mutants, suggesting that RNA synthesis by Pol V is also unimpaired.

MRD1 is representative of a small set of *Arabidopsis* loci at which RdDM and silencing requires Pol IV but not Pol V. Thus, *MRD1* is expressed at low levels in Pol V mutants (*nrpe1-11)*, as in wild-type plants, but is substantially derepressed in Pol IV mutants (*nrpd1-3)*, unlike AtSN1 or soloLTR elements that require both Pol IV and Pol V for silencing (Figure 4A). In *9a-1* or *9b-1* mutants, *MRD1* is not derepressed, suggesting that Pol IV function is not impaired in either single mutant (Figure 4A, lanes 4 and 5).

Pol V Transcripts Are Produced in 9b Mutants

Pol V transcripts can be detected at specific intergenic loci such as *IGN5* (Law et al., 2010; Wierzbicki et al., 2008; Wierzbicki et al., 2009), therefore we examined whether *9b-1* mutants are impaired for Pol V transcription. *IGN5* transcripts are readily detected in wild-type plants or Pol IV mutants (*nrpd1*) but are substantially reduced in *nrpe1* mutants (Figure 4B). *IGN5* transcript abundance is not affected in the *9b-1* mutant (Figure 4B), suggesting that Pol V's ability to synthesize RNA is not impaired and that a step downstream of RNA synthesis might be impaired, instead.

DISCUSSION

In mutants lacking the 9b subunit, Pol IV-dependent siRNA biogenesis is not impaired, nor is silencing of *MRD1*, a locus whose RdDM and repression is dependent on Pol IV but not Pol V. However, loci that require both Pol IV and Pol V for silencing are derepressed in *9b-1* mutants. Based on these observations, we deduce that loss of silencing in *9b-1* mutants is due to a defect in Pol V function. Interestingly, Pol V transcription does not appear to be impaired in *9b-1* mutants, based on *IGN5* transcript production and siRNA abundance at loci where siRNA levels depend, in part, on Pol V activity. Therefore, we reason that the impairment of Pol V function in 9b mutants is not due to a decreased ability of Pol V to synthesize RNA, but an impairment of a regulatory function, possibly mediated by interactions with other proteins. Consistent with this hypothesis, the eight amino acids that are different in the 9a and 9b subunits are predicted to be exposed on the surface of the proteins, based on their homology to yeast Rpb9 (Figure 4C), whose structure is known. Figure 4D shows a space filling model in which the predicted positions of the polymorphic amino acids of 9a and 9b are mapped onto the corresponding amino acid positions of Rpb9 within a yeast Pol II elongation complex (PDB:1Y1W) (Kettenberger et al., 2004).

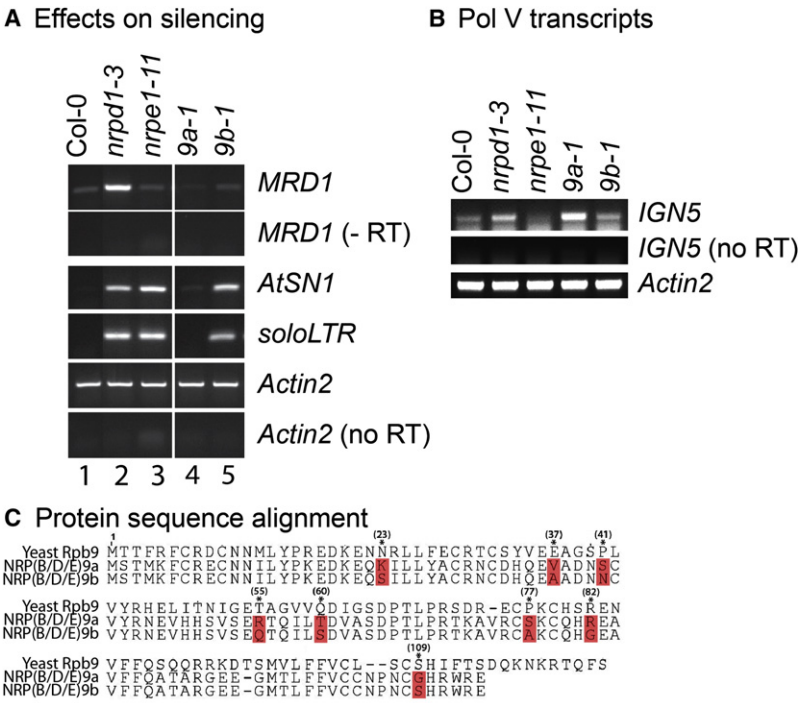

Figure 4. NRPB9b Is Not Required for Pol V-Dependent Transcription or Pol IV-Mediated Silencing

(A) Pol IV-dependent, but Pol V-independent, silencing of locus *MRD1*. RNA isolated from wild-type (Col-0), *nrpd1-3, nrpe1-11*, *9a-1*, or *9b-1* mutants was subjected to RT-PCR using primers specific for *MRD1*, soloLTR, or AtSN1.

(B) Detection of Pol V transcripts at IGN5 using RT-PCR. Col-0, *nrpd1-3*, *9a-1*, *9b-1*, and *nrpe1-11* are compared.

(C) Amino acid sequence alignment of yeast Rpb9 with *Arabidopsis* NRP(B/D/E)9a and NRP(B/D/E)9b. Amino acids that differ in the 9a and 9b subunits are highlighted. Numbers correspond to amino acid positions of the *Arabidopsis* 9a and 9b proteins, and not (necessarily) to yeast Rpb9.

(D) Locations of the eight amino acids that are polymorphic in the *Arabidopsis* 9a and 9b subunits, mapped (in red) onto a space-filling rendering of yeast Rpb9 (green) within a Pol II elongation complex (PDB:1Y1W). Rpb1 is colored gray, Rpb2 is blue, Rpb5 is gold, and the DNA is pink. The image on the right is rotated clockwise relative to the image on the left to show the position of the DNA duplex. Rpb9 amino acids colored red correspond to the amino acids that align with the polymorphic amino acids of the *Arabidopsis* 9a and 9b subunits, and are numbered based on the *Arabidopsis* sequences.

Rpb9 has two zinc finger domains, Zn1 and Zn2, which are located near the N and C termini of the protein. The Zn2 domain shares homology with the elongation/transcript cleavage factor, TFIIS and is thought to catalyze transcript cleavage events in partnership with TFIIS. Transcript cleavage is important for RNA 3′-end processing, transcription termination, and polymerase backtracking that allows for correction of misincorporated nucleotides, escape from an arrested state, or DNA repair at damaged sites (Awrey et al., 1997; Hemming et al., 2000; Koyama et al., 2007; Nesser et al., 2006; Walmacq et al., 2009). The Rpb9-paralogous subunits of Pols I and III, Rpa12, and Rpc11, also possess transcript cleavage activity and are stronger endonucleases than Rpb9, suggesting that Rpb9 has evolved to be regulated by TFIIS or other factors (Ruan et al., 2011). Several of the amino acid differences between NRP(B/D/E)9a and NRP(B/D/E)9b occur within the Zn2 domain (amino acids 77, 82, and 109). *Arabidopsis* has multiple genes encoding TFIIS-like proteins, leading us to speculate that the eight amino acids that differ between the 9a or 9b proteins might specify interactions with different TFIIS-like proteins, or with proteins that mediate chromatin modifications at Pol V-transcribed loci.

The single multisubunit RNA polymerases used by archaea closely resemble eukaryotic RNA Polymerase II except that they lack an Rpb9-like subunit (Werner and Grohmann, 2011); likewise, yeast strains deleted for the *RPB9* gene are viable (Woychik et al., 1991). These observations have suggested that Rpb9 is an important, but nonessential, regulatory subunit in eukaryotes. However, our results show that NRPB9 function is essential in *Arabidopsis*. Either NRPB9a or NRPB9b (the subunits named in the context of Pol II) is sufficient for viability, but embryogenesis cannot be completed in *nrpb9a nrpb9b* double mutants.

It is noteworthy that *nrpb9a nrpb9b* mutants develop further than do null mutants for catalytic subunits of Pols I, II, and III, in which female gametophytes fail to develop and are never fertilized, such that no embryogenesis takes place (Onodera et al., 2008). Therefore, NRPB9-mediated functions may be partially dispensable in plants, as in yeast and archaea, specifically at the haploid gametophytic stage of the plant life cycle. However, NRPB9 function is essential during the diploid sporophyte stage of the plant life cycle, at one or more steps required for embryo development, beginning at the globular embryo stage. NRPB9 functions must also affect later vegetative development in order to explain the distinct phenotypes of *nrpb9b* mutants.

Our data suggest that Pol IV functions are not impaired in *9a-1* or *9b-1* mutants. One possibility is that the ninth subunit is

dispensable for Pol IV function. Alternatively, the 9a and 9b subunits might be redundant in the context of Pol IV, as they are for most Pol II functions. Unfortunately, the lethality of the *9a-1* or *9b-1* double mutant precludes an easy test of whether a functional ninth subunit is required for Pol IV activity.

Spectral counts of peptides detected upon mass spectrometric analyses of affinity purified Pol V suggest that the NRPE9a and NRPE9b-containing Pol V subtypes coexist in similar abundance. Our study elucidates a functional requirement for the NRPE9b-containing Pol V subtype (Pol V_{9b}), but not the NRPE9a-containing form of Pol V (Pol V_{9a}). Interestingly, affinity purification of the DDR complex (DRD1, DMS3, and RDM1), which is required for Pol V transcription at *IGN5* and other loci (Law et al., 2010; Wierzbicki et al., 2009), resulted in copurification of Pol V subunits that included NRPE9a (Law et al., 2010). One possibility is that Pol V_{9a} functions only at specific loci. Alternatively, Pol V_{9a} and Pol V_{9b} may both be engaged at most, or all loci subjected to RdDM. If so, the genetics indicate that Pol V_{9b} is comprehensive in its functions, explaining the dispensability of the 9a gene (and Pol V_{9a}), whereas Pol V_{9a} has more limited functionality. By the same logic, Pol II_{9b} must have broader functionality than Pol II_{9a} in order to explain the mutant vegetative phenotypes of *9b-1* mutants, the wild-type phenotypes of *9a-1* mutants, but the lethality of the double mutant.

EXPERIMENTAL PROCEDURES

Plant Material

nrp(b/d/e)9a-1 (Salk_032670) and *nrp(b/d/e)9b-1* (Salk_031043) seeds were obtained from the *Arabidopsis* Biological Resource Center. Plants were grown on soil using an 18 hr light/6 hr dark cycle. Genotyping PCR primers flanking the T-DNA insertions (Salk032670_LP: 5′CAGACAAAGAACAGTGTCATTCC, Salk032670_RP: 5′TTCTGGAATTGCACCTCTCTG, Salk031043_LP: 5′GATA TAAAGGTGCATGGGGATATGC, Salk031043_RP: 5′TAAACTCATTAAATTA TCATTCCTTGG) were used in conjunction with a T-DNA left border primer (LBa1: TGGTTCACGTAGTGGGCCATCG).

RT-PCR Assays

Total RNA was purified from leaves of 3-week old plant using Trizol reagent (Invitrogen). RQ1 DNase-treated RNA (100 ng) (Promega) was reverse transcribed using SuperScriptIII (Invitrogen) and gene specific primers. PCR of resulting cDNA was performed using HotStart Taq (Fermentas). Primers for *soloLTR*, *ATSN1*, and *Actin* were previously described (Wierzbicki et al., 2008). Primers for *NRPB9a* and *NRPB9b* transcripts amplified 5′ untranslated regions (UTR): 9a_5′UTR: GTGATTCAGTTTTGGTTTTGGAAC CTAA, 9b_5′UTR: GTGAAATCAAAGAAGCATTCAAAAGCTC, 9aRev: TTCT CTCCAGCGATGACCAC, and 9bRev: TTCTCTCCAACGGTGACTACAGTT.

DNA Methylation Assays

DNA was extracted using a Nucleon PhytoPure Genomic DNA Extraction Kit (GE Healthcare). DNA (1 ug) was subjected to restriction endonuclease digestion, agarose gel electrophoresis, and Southern blot hybridization using a *5S* rRNA gene probe as described in (Blevins et al., 2009). For methylation-sensitive PCR assays, 1 ug of DNA was digested overnight with the appropriate restriction enzyme (New England Biolabs) and 50 ng of DNA was subjected to PCR using GoTaq Green polymerase (Promega) and flanking primers (Wierzbicki et al., 2008).

Small RNA Blots

RNA was purified from inflorescence tissue using an RNeasy Kit (QIAGEN). Low-molecular-weight RNA (4 ug) was subjected to electrophoresis on a 12% denaturing polyacrylamide gel and transferred to Hybond membranes (GE Healthcare). Prehybridization and hybridization in PerfectHyb buffer (Sigma) was performed at 37°C. DNA oligonucleotide probes were ^{32}P end-labeled using T4 Polynucleotide Kinase: siR1003: ATGCCAAGTTTGGCCT CACGGTCT, soloLTR: TGTCATTATCCATCATTCATCTCTATCCATAAG, and miR160: TGGCATACAGGGAGCCAGGCA.

Cloning and Complementation

A genomic clone of *NRP(B/D/E)9b* was obtained by PCR amplification of *A. thaliana* genomic DNA using PFU Ultra DNA polymerase (Stratagene) and primers: 9bPromF: CACCGCACTTCAACAACCCAATTACA and 9bRev: TTC TCTCCAACGGTGACTACAGTT. PCR products captured in pENTR D/TOPO (Invitrogen) were recombined into pEARLEYGATE 302 (Earley et al., 2006) using LR CLonase II (Invitrogen) and transformed into *nrp(b/d/e)9b-1* homozygous mutants using the floral dip method for *Agrobacterium*-mediated gene transfer (Clough and Bent, 1998).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.celrep.2012.01.004.

LICENSING INFORMATION

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